SEROLOGICAL INVESTIGATION OF DROSOPHILA ANTIGENS WITH THE COMPLEMENT FIXATION REACTION

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Introduction

This investigation had its origin in a zoölogy seminar in January, 1936. The unpublished immunological works of Irwin and his associates regarding dove-pigeon hybrids, were under discussion. The suggestion was made that perhaps a similar study of *Drosophila* species would be of interest to geneticists and taxonomists. The question was discussed with Professor V. T. Schuhardt, who suggested that the ring-precipitin and complement fixation reactions should be tried. As a consequence of this conversation, Professor Schuhardt and R. W. Cumley initiated an immunization program, in order to determine roughly whether the formation of antibodies could be stimulated in rabbits in response to the injection of a *Drosophila* antigen. A rabbit was injected intraperitoneally, at three-day intervals, with 4 cc. saline suspensions of macerated bodies of *Drosophila melanogaster*. In the subsequent tests the complement fixation reaction was employed; and in these tests the antiserum to *Drosophila melanogaster* antigen yielded complement fixing antibodies in an antiserum dilution of 1:320, when tested against its homologous antigen. Heterologous test antigens made from two other species of *Drosophila* gave titres of 1:160 and 1:80, respectively.

During the following spring and summer T. A. Koerner and R. W. Cumley inoculated several rabbits intravenously with suspensions of macerated flies, in dilutions of 1:100, and in doses ranging from 1.0 to 25.0 cc. Several rabbits died, and no significant results were obtained. These initial studies were of considerable importance, however, since they defined certain limits regarding the size of the antigenic dose.

In July, 1936 Levit *et al.* announced their results in detecting the presence of the Y-chromosome in males and attached–X

females of *Drosophila melanogaster,* through the use of complement fixation methods (Levit, Ginsburg, Kalinin, and Feinberg, 1936). Although they did not present the details of their technics, they confirmed the belief in this laboratory that antibodies to *Drosophila* could be demonstrated.

In October, 1936 R. W. Cumley and Sol Haberman* initiated another series of inoculations. The males and females of the following species were separated: *Drosophila bipectinata, D. caribbea, D. hydei, D. melanogaster, D. mulleri,* and *D. virilis.* The flies were macerated, and saline was added in the proportion of one gram of the fresh fly material to 50 cc. of 0.85% NaCl. After the mixture remained for two days in the ice box, it was filtered through several thicknesses of filter paper. Rabbits were injected intravenously at about three-day intervals with a total of eleven doses of this filtered broth. The doses were of increasing size, beginning with 0.2 cc. of a 1:1000 dilution and proceeding to 0.4 cc. of a 1:50 dilution. Subsequent tests showed that no appreciable antibody formation occurred from the injection of such small doses. The animals were then inoculated with five larger doses, in accordance with Professor Schuhardt's views, and as had been suggested by the first inoculations which he and Cumley had made. These doses ranged from 2 cc. of a 1:50 dilution to 4 cc. of a 1:50 dilution. A trial bleeding of a few cubic centimeters was made six days after the last injection. The blood showed the presence of complement-fixing antibodies, and the animals were bled from the heart two days later.

In the subsequent complement fixation and precipitation tests low dilutions of complement-fixing antibodies and confusing results were the rule. From these experiments the conclusions were drawn that (1) more exact methods of standardization of antigens should be used; and (2) a more concentrated antigen, prepared from dried flies, would be necessary. This last conclusion is contrary to the findings of Brown and Heffron (1928), in their serological investigations of Lepidoptera; they reported that precipitating antibodies were produced in greater quantity when the fresh material, rather than the dried, was used as antigen.

* Mr. Haberman served in the capacity of technical assistant, and was paid from the research fund of the Department of Zoology.

Materials and Methods

The results reported in this paper were obtained from testing the sera of rabbits which had been immunized to saturated saline solutions of dried flies. Martin and Cotner (1934) successfully immunized rabbits to moth species by using similarly prepared antigens. In the present experiments the following species of *Drosophila* were employed: *bipectinata, caribbea, hydei, melanogaster,* and *virilis.* The flies were grown in half-pint milk bottles, on the yeast-banana agar used in routine genetical experiments. Usually, the flies were removed from the food within two days after hatching. After being weighed, they were macerated in a mortar. The pasty material contained in the mortar was desic-

TABLE 1

Sample	*Drosophila* Species	Weight before drying	Weight after drying	Per cent water	Per cent dry powder
1	*virilis*	17.76 grams	5.74 grams	67.7	32.3
23	*virilis*	23.78 "	6.34 "	73.3	26.7
25	*virilis*	41.36 "	9.27 "	77.6	22.4
2	*caribbea*	27.04 "	6.92 "	74.4	25.6
7	*caribbea*	25.93 "	6.82 "	73.7	26.3
22	*caribbea*	19.89 "	4.76 "	76.1	23.9
24	*caribbea*	37.15 "	8.75 "	76.4	23.6
3	*hydei*	8.63 "	2.30 "	74.3	25.7
10	*hydei*	11.86 "	3.28 "	72.4	27.6
18	*hydei*	21.31 "	4.84 "	77.3	22.7
4	*melanogaster*	21.15 "	6.12 "	71.1	28.9
16	*melanogaster*	31.86 "	8.17 "	74.4	25.6
26	*melanogaster*	11.40 "	3.32 "	70.9	29.1
5	*mulleri*	22.65 "	8.19 "	63.9	36.1
11	*mulleri*	9.25 "	3.37 "	63.5	36.5
15	*mulleri*	6.83 "	2.26 "	66.9	33.1
20	*mulleri*	11.25 "	3.13 "	72.3	27.7
6	*sulcata*	9.55 "	3.14 "	67.0	33.0
12	*bipectinata*	12.62 "	3.44 "	72.7	27.3
17	*bipectinata*	32.63 "	7.67 "	76.5	23.5
21	*bipectinata*	23.92 "	5.67 "	76.3	23.7
14	*funebris*	29.17 "	8.43 "	71.1	28.9

cated *in vacuo* over sulfuric acid for two days. The material was removed, ground still more, and returned to the desiccator for further drying. When the powder was thoroughly dry, it was removed and weighed. No attempt was made to insure complete removal of the water, since the nitrogen content of the samples was to determine their ultimate standardization. Table 1 shows data regarding the weights of several species of *Drosophila* before and after drying. The percentages of water removed in desiccation, by this method, varied from 63.5% to 77.6%.

The powdered flies obtained by desiccation and grinding were mixed with 0.85% NaCl in the ratio of 1 gram of fly powder to 10 cc. of saline. The mixture was allowed to remain in the ice box at about 9 degrees Centigrade for two days. The broth was then centrifuged and the clear supernatant solution decanted. This solution was filtered through several thicknesses of filter paper and preserved with Merthiolate Solution (1:10,000). Table 2 presents data relating to the preparation of the saline extracts. From this table one may note that the amount of fly powder which will go into solution per cubic centimeter of saline varies considerably from sample to sample. In order to have standardized the antigens on the basis of weights of materials, one would have had to consider the weight of the material extracted.

The clear saline extract taken directly from the powder was used for immunization of the animals. Rabbits were inoculated seven times with doses increasing from 1 cc. to 4 cc. of the above described solution. These injections were rather irregular, since several trial bleedings were made at intervals to determine the presence of complement-fixing antibodies. The inoculations made several of the rabbits extremely sick, and six of them died in the course of the immunization. Seven days after the seventh injection the rabbits were bled from the heart, without anaesthesia, The amount of blood taken from each rabbit varied from 8 cc. to 30 cc.

In the complement fixation reactions serial dilutions of the antisera were made, and all the antigens were tested against a particular antiserum. No attempt was made to standardize the protein content or antibody content of the antisera. The test antigens, however, were standardized. Micro-Kjeldahl tests were

TABLE 2

Sample number	*Drosophila* Species	Grams of dried powder	Cc. of saline added	Cc. of extract removed	Grams of dissolved material in extract	Grams of undissolved residue	Dilution of antigen extract: gm./cc.
1	*virilis*	3.00	30.00	23.00	0.655	2.345	1: 34.5
1	*virilis*	2.74	27.40	21.00	0.790	1.950	1: 26.6
2	*caribbea*	3.00	30.00	22.00	0.835	2.165	1: 26.4
3	*hydei*	2.29	22.90	16.00	0.755	1.540	1: 21.2
10	*hydei*	3.27	32.70	24.00	1.275	2.000	1: 18.8
4	*melanogaster*	4.00	40.00	30.00	0.975	3.025	1: 30.8
5	*mulleri*	4.00	40.00	34.00	1.875	2.125	1: 18.1

run on each of the various antigen extracts, and the solutions were adjusted to have equivalent nitrogen contents. The nitrogen contents of the saline extracts of dry flies and the saline extracts of wet flies, mentioned earlier in this paper, are shown in Table 3. One may observe that the nitrogen contents of the samples vary considerably, rendering the adjustment for nitrogen equivalence imperative. As is revealed in this table, the nitrogen

TABLE 3

Fresh flies. Extracted 1: 100 in saline

Drosophila Species	Mgm. of nitrogen per cc. of extract
melanogaster	0.0470
virilis	0.0420
simulans	0.0429
pseudoobscura A	0.0384
miranda	0.0464

Dried flies. Extracted 1: 50 in saline

Drosophila Species	Mgm. of nitrogen per cc. of extract
melanogaster	1.2200
mulleri	0.6960
hydei	0.6370
virilis	0.7280
caribbea	0.6870
bipectinata	1.1680

contents of the 1: 50 extracts of the dried flies are from fifteen to twenty-five times as great as those of the 1: 100 extracts of the fresh flies, a fact which probably accounts for the increased antigenicity of the dried fly extracts.

The antigens which were to be used for the tests were first tested to determine whether they possessed properties which would inhibit or interefere with the normal action of the complement, *i.e.*, whether the *Drosophila* antigens possessed active anticomplementary agents. The results of this test are shown in Table 4. Since a two-plus (++) reaction took place in the 1: 2400 dilution of the *D. hydei* antigen, all the antigens were diluted to

TABLE 4

Type of Antigen	Dilution of Antigen										
	1: 50	1: 100	1: 150	1: 200	1: 300	1: 400	1: 600	1: 800	1: 1200	1: 1600	1: 2400
Melanogaster	++	−	−	−	−	−	−	−	−	−	−
Mulleri	++++	+++	+++	++	+++	++	+	+	±	±	±
Virilis	++++	++	+	−	−	−	−	−	−	−	−
Caribbea	+++	++	+	±	±	±	−	−	−	−	−
Hydei	++++	++++	++++	++++	++++	++++	++++	+++	+++	++	++
Bipectinata	++++	++++	++++	++++	+++	+++	++	+	−	−	−

1:5000 before proceeding with complement fixation tests. Consequently, the antigens were diluted to a point considerably beyond the range in which anticomplementary activity occurred.

The standard complement fixation reaction was used in testing for the presence of complement-fixing antibodies in the sera of immunized rabbits. The antisera were diluted serially, and the antigens were held constant. Complete hemolysis in any given tube was recorded as negative (−). Complete lack of hemolysis was recorded as positive (++++) complement fixation. Three intermediate grades of fixation were recorded as one-plus (+), two-plus (++), and three-plus (+++), on the basis of relative amounts of hemolysis.

RESULTS

In order to determine roughly the end-points of complement fixation, a series of tests were run in which the various antisera were tested against their homologous antigens. The results of these tests are shown in Table 5. From the titres recorded in these tests, the dilutions of antisera which were to be used in heterologous tests were indicated. The heterologous tests yielded the results that are shown in Table 6. From this table the following features may be noted:

1) There is great variability in antibody production by rabbits immunized to the same antigen. Not only do different rabbits yield sera which differ in their antibody content, but the order in which the various antigens react with a given serum varies slightly from one rabbit to another. This may be attributed to individual differences of rabbits, to errors in reading the tests, and to errors inherent in the use of unpurified antigens.

2) There is a lack of reciprocal relations between antisera and antigens. In general, the order of relationships is the same, but the percentage relationships, as revealed in Table 7, varies considerably in the reciprocal tests. This question of reciprocity will be dealt with in detail in a subsequent paper. Reciprocity is not believed to be a necessary adjunct to the antigen-antibody relationship.

Table 7 presents the antigen-antiserum relationships that have been calculated from the data of Table 6. In calculating these relationships, modifications of the methods of Boyden (1926,

TABLE 5

Type of Antiserum	Type of Antigen	Dilution of Antiserum									
		1: 30	1: 40	1: 60	1: 80	1: 120	1: 160	1: 240	1: 320	1: 480	1: 640
Mulleri 3	Mulleri	++++	++++	++++	+++	+	−	−	−	−	−
Mulleri B5	Mulleri	++++	++++	++++	++	±	−	−	−	−	−
Mulleri 4	Mulleri	++++	+++	+	+	−	−	−	−	−	−
Mulleri B6	Mulleri	++++	++++	+	+	−	−	−	−	−	−
Virilis A4	Virilis	++++	++++	++++	+	−	−	−	−	−	−
Caribbea C1	Caribbea	++++	++++	++++	+	+	−	−	−	−	−
Caribbea C4	Caribbea	++++	++++	++++	++++	+	+	−	−	−	−
Melanogaster A1	Melanogaster	++++	++++	+++	+++	++	++	−	−	−	−
Melonagaster B1	Melanogaster	++++	+++	+	−	−	−	−	−	−	−

TABLE 6

Type of Antiserum	Type of Antigen	Dilution of Antiserum										
		1:10	1:20	1:30	1:40	1:50	1:60	1:80	1:100	1:120	1:160	1:200
Mulleri 3	Mulleri		++++	++++	++++	++++	++	+	–	–	–	–
	Virilis		++++	+++	+	+	–	–	–	–	–	–
	Hydei		++++	++	–	–	–	–	–	–	–	–
	Caribbea		++++	+	–	–	–	–	–	–	–	–
	Bipectinata		+	–	–	–	–	–	–	–	–	–
	Melanogaster		–	–	–	–	–	–	–	–	–	–
Mulleri B5	Mulleri	++++	++++	++++	++++	++++	++++	+++	++	+++	+++	
	Virilis	++++	++++	++++	++++	++++	+++	++	+	+	–	
	Hydei	++++	++++	++++	++++	++++	+++	++	+	+	–	
	Caribbea	++++	++++	++++	++++	+++	+++	++	+	–	–	
	Bipectinata	++++	++++	++++	++++	+++	++	+	–	–	–	
	Melanogaster	++++	++++	++++	+++	++	++	+	–	–	–	
Mulleri 4	Mulleri	++++	++++	++++	+++	+	+	+	–	–	–	
	Hydei	++++	++++	++++	++++	+++	+++	–	–	–	–	
	Caribbea	++++	++++	++	–	–	–	–	–	–	–	
	Virilis	++++	+++	+	–	–	–	–	–	–	–	
	Bipectinata	–	–	–	–	–	–	–	–	–	–	
	Melanogaster	–	–	–	–	–	–	–	–	–	–	
Mulleri B6	Mulleri	++++	++++	++++	++++	++	+	+	–	–	–	
	Virilis	++++	++++	+	–	–	–	–	–	–	–	
	Caribbea	++++	+++	+	–	–	–	–	–	–	–	
	Hydei	++++	++	–	–	–	–	–	–	–	–	
	Bipectinata	++	+	–	–	–	–	–	–	–	–	
	Melanogaster	–	–	–	–	–	–	–	–	–	–	
Virilis A4	Virilis	++++	++++	++++	+++	+++	+	–	–	–	–	
	Mulleri	++++	++++	+++	++	+	–	–	–	–	–	
	Caribbea	++++	++++	++++	++	–	–	–	–	–	–	
	Hydei	++++	++++	++	–	–	–	–	–	–	–	
	Bipectinata	++	–	–	–	–	–	–	–	–	–	
	Melanogaster	+	–	–	–	–	–	–	–	–	–	

TABLE 6—(*Concluded*)

Type of Antiserum	Type of Antigen	Dilution of Antiserum										
		1: 10	1: 20	1: 30	1: 40	1: 50	1: 60	1: 80	1: 100	1: 120	1: 160	1: 200
Caribbea C1	Caribbea	++++	++++	++++	++	+	−	−	−	−	−	
	Mulleri	++++	+++	+	+	−	−	−	−	−	−	
	Virilis	++++	++	+	+	−	−	−	−	−	−	
	Hydei	++++	++	+	−	−	−	−	−	−	−	
	Bipectinata	+++	++	+	−	−	−	−	−	−	−	
	Melanogaster	+++	+	+	−	−	−	−	−	−	−	
Caribbea C4	Caribbea		++++	++++	++++	+++	++	++	+	−	−	−
	Mulleri		+++	++	+	+	−	−	−	−	−	−
	Virilis		+++	+++	+	−	−	−	−	−	−	−
	Bipectinata		+++	+	+	−	−	−	−	−	−	−
	Hydei		+++	+	−	−	−	−	−	−	−	−
	Melanogaster		++	−	−	−	−	−	−	−	−	−
Melanogaster A1	Melanogaster		++++	++++	+++	+	+	+	−	−	−	−
	Caribbea		++++	+++	++	+	+	−	−	−	−	−
	Hydei		++++	+++	++	+	+	−	−	−	−	−
	Mulleri		++	++	+	−	−	−	−	−	−	−
	Bipectinata		+	+	−	−	−	−	−	−	−	−
	Virilis		+	−	−	−	−	−	−	−	−	−
Melanogaster B1	Melanogaster	++++	+++	+	−	−	−	−	−	−	−	
	Caribbea	+++	+++	+	−	−	−	−	−	−	−	
	Mulleri	++	+	+	−	−	−	−	−	−	−	
	Virilis	++	+	−	−	−	−	−	−	−	−	
	Hydei	+	−	−	−	−	−	−	−	−	−	
	Bipectinata	−	−	−	−	−	−	−	−	−	−	

TABLE 7

Type of Antiserum	Type of Antigen	Percentage Antigen-Antiserum Relationship: highest dilution of reaction (Boyden)	Percentage Antigen-Antiserum Relationship: total number of pluses (Nelson & Birkeland)
Mulleri	Mulleri	100.00	100.00
	Virilis	55.20	55.10
	Hydei	55.20	48.30
	Caribbea	44.80	47.10
	Bipectinata	Less than 38.90	Less than 29.00
	Melanogaster	Less than 50.00	Less than 50.00
Virilis	Virilis	100.00	100.00
	Mulleri	83.40	70.00
	Caribbea	66.60	70.00
	Hydei	50.00	50.00
	Bipectinata	16.70	10.00
	Melanogaster	16.70	5.00
Caribbea	Caribbea	100.00	100.00
	Mulleri	65.00	47.50
	Virilis	60.00	44.20
	Bipectinata	50.00	35.90
	Hydei	45.00	33.40
	Melanogaster	40.00	21.70
Melanogaster	Melanogaster	100.00	100.00
	Caribbea	87.50	80.60
	Mulleri	75.00	42.90
	Hydei	54.20	45.60
	Virilis	45.80	22.32
	Bipectinata	Less than 37.50	Less than 14.30

1932, 1934) and of Nelson and Birkeland (1929) have been used. These authors worked with precipitation technics; their computing formulæ have been applied herein to the complement fixation reaction. The values were calculated on the basis of the highest dilution of reactivity and on the basis of the total number of pluses, *i.e.,* the strength of reactivity. These figures should not be interpreted as representing the relations that exist between the fly species, or as representing the actual percentage of likeness or unlikeness between any two species. Rather they show the extent to which several antigens react with a given antiserum, when compared with the homologous antigen-antibody reaction. For example, the *virilis* antigen reacts with the *virilis* antiserum at a dilution arbitrarily designated as 100%, whereas *mulleri*

antigen reacts at a dilution only 83.4% as great, and *melanogaster* antigen reacts at a dilution only 16.7% as great as the dilution at which the *virilis* antigen reacted. The chief value of these percentage relationships is that they indicate the serological ranks assumed by the various antigens.

Discussion

The reliability of these data is dependent upon several factors, of which a few will be considered. The standardization of the various reagents is of paramount importance. In this work the antigens were standardized in the manner previously mentioned. The amboceptor and complement were properly titrated, and controls for the amboceptor, complement, and sheep cells were carried with each set of tests. The antisera were considered variables; the antigens were accepted as constants. Several authors have suggested more exact standardization procedures, including lipoid extraction of antigens (Boyden 1936; Moritz 1934; Becker 1932), globulin extraction of antigens (Nelson and Birkeland 1929), determination of protein and nonprotein nitrogen content of antigens (Boyden 1934; Eisenbrandt 1936), and the use of buffered saline (Boyden 1926). The value of these presumably more exact procedures becomes evident only after tests have been made with the native unaltered antigens. This paper treats only of these latter materials. Other methods will be discussed in later publications.

As an antigen *Drosophila* presents inherent difficulties which possibly bear upon the reliability of these tests. The intestinal contents of the flies should be eliminated from the antigens. Several months of intensive effort failed to yield bacteria- and yeast-free flies in quantities large enough for making antigens. Some success has been attained in producing flies relatively free of food and yeasts.

Another factor which should influence the reliability of these tests is the possible presence of natural antibodies to *Drosophila* in the serum of the rabbits. All of the rabbits used in this investigation were found to be free of any such natural antibodies. Furthermore, three control animals were tested. Two of these animals had never been immunized to foreign material. The third

had been immunized previously to staphylococci. The two unimmunized animals showed no complement-fixing antibodies; while the third showed the presence of complement-fixing antibodies in low dilutions. This may be explained as a nonspecific reaction due to sharing of antigen complexes or to the presence of similar antigenic factors in both *Staphylococcus* and *Drosophila,* or to the presence of staphylococci in or on the drosophilas used as the test antigen.

CONCLUSION

The complement fixation reaction can be used in differentiating the antigens of various *Drosophila* species. The results of the present investigation, although not entirely consistent, reveal roughly the serological ranking of the various species under consideration. Greater reliability probably will proceed from the use of more purified and better standardized reagents than were employed in the tests reported herein. Other experiments are now in progress which make use of more refined procedures.

SUMMARY

1. Methods and data regarding the preparation of *Drosophila* antigens and antisera were offered.

2. The complement fixation reaction was used in comparing the reactivity of several antigens to different antisera, and the results were presented in Table 6.

3. From the data in Table 6, the percentage of antigen-antibody reactivity was calculated. These calculations were based upon the highest antiserum dilution at which complement was fixed, and upon the total number of +'s recorded in the tests. These methods are modifications of the Boyden and of the Nelson and Birkeland computation technics. The new calculated values were recorded in Table 7.

4. The values in Table 7 were indicated as revealing the ranks assumed by the various antigens with reference to a given antiserum.

5. A discussion was given of the reliability of the complement fixation reaction in differentiating *Drosophila* species.

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